Evaluation of a library of FDA-approved drugs for their ability to potentiate antibiotics against multidrug resistant Gram-negative pathogens

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Abstract

The Prestwick library was screened for antibacterial activity or 'antibiotic-resistance breaking' (ARB) potential against four species of Gram-negative pathogens. Discounting known antibacterials, the screen identified very few ARB hits, which were strain/drug specific. These ARB hits included antimetabolites (zidovudine, floxuridine, didanosine, gemcitabine), anthracyclines (daunorubicin, mitoxantrone, epirubicin) and psychoactive drugs (gabapentin, fluspirilene, oxethazaine). This suggests that there are few approved drugs which could be directly repositioned as adjunct-antibacterials and these will need robust testing to validate efficacy.

Main text

The need for new antibiotics is driven by the rapid spread of multidrug resistant (MDR) bacterial pathogens and the absence of new antibiotics in the clinical development pathway is significant cause for concern. The idea of repurposing existing drugs, which are currently used as treatments for other disease areas is attractive because, due to the known safety profile of approved drugs, the cost and time to clinic could be significantly lower than novel scaffolds [1]. Examples of successful repurposing screens, outside of the antibacterial area, have produced candidates for Ebola, Zika virus and anti-cancer therapies [2-4]. Recent studies for the identification of new antibacterial leads have focussed on two key areas; i) identification of direct antibacterial hits for one or more target bacteria [5, 6], and ii) screening for compounds which synergise with existing antibiotics, thereby restoring activity of the antibiotic against strains/species which are currently resistant to their use [7]. Several previous studies identified antibacterial activities that are too weak to be effective on their own and would require exposures greater than the maximum concentration achievable with their primary pharmacology and recommended safe dosing [7], possibly because of the bacterial membrane barriers.

The current study aimed to identify either direct-acting antibiotics, or compounds which sensitise resistant Gram-negative strains to one or more antibiotics, looking to identify 'Antibiotic Resistance Breakers' (ARBs).

A high-throughput combination screen (HTCS) of potential ARBs and antibiotics was performed in 384-well format from the Prestwick library of 1280 selected compounds in combination with five antibiotics or 0.1 % DMSO, in duplicate. Each replicate was from independent dilution plates by using independent inocula on two different days. The potential ARBs were tested at two concentrations, 20 µM and 7 µM, in combination with antibiotics at 0.125 x MIC. Concentrations were selected to balance the probability of achieving a significant number of hits with realistic concentrations which align with the likely Cmax for a typical drug. Where the MIC was >128 mg/L, the antibiotic was tested at 16 mg/L. The MICs of test articles were determined in cation-adjusted

Mueller-Hinton broth (caMHB; Oxoid), using the Clinical and Laboratory Standards Institute (CLSI) guidelines M7-A10 & M100-S26.

Clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii* which were recently highlighted by the World Health Organisation as priority pathogens for which new antibiotics are urgently required [8], were selected which were resistant to each antibiotic. In some species (*K. pneumoniae* and *A. baumannii*), this involved use of two strains to cover all resistance profiles, and some resistance profiles were not available (Table S1).

During the HTCS, bacterial growth was determined by reading on a modal reader (Infinite 500, Tecan) at 600 nm after 24 h of incubation. For each plate, OD600 measurement was done at 2 timepoints, T0 h (to determine the background signal related to the coloured compounds) and T24 h at the end of incubation. After blank substitution, calculated by subtracting OD600 at T0 h from OD600 at T24 h, a normalization step was carried out between OD600 values obtained in wells containing the compounds compared to those obtained in control wells (DMSO wells – maximal growth). Data analysis for each run was performed with Genedata Screener software. The workflow from the raw data associated to plate-map up to the normalization step was fully automated allowing for complete tracking of all data. The Z' factor and assay window were determined for each plate, between the positive control in presence of antibiotic at 0.125 x MIC and the negative control [9]. The Z' factor for each combination of strain and antibiotic was between 0.5 – 0.8, plates displaying a Z' factor < 0.5 were automatically retested.

After statistical analysis, hits were defined as data points with an activity > hit threshold based on the Sigma method (mean + 3 standard deviations), unless otherwise stated. Results were expressed as percentage of growth inhibition compared to that in untreated controls (exposed to 0.1% DMSO only), assessed by optical density.

Firstly, compounds from the library were tested for direct antimicrobial activity at two concentrations, 7 μM and 20 μM, in the presence of 0.1 % DMSO (Figure S1 and S2). The number of direct hits at either concentration varied considerably between species, with 29 for *E. coli,* 16 for *P. aeruginosa,* 85 for the two *A. baumannii* strains combined and 53 for the two *K. pneumoniae* strains (discounting overlapping hits between the two strains of the same species and between the two concentrations tested) (Table S2). As might be expected we saw three scenarios with respect to dose response, i) compounds which were equally effective at both concentrations, ii) compounds which were effective at 20 μM which were not effective as either direct antibacterials or ARBs at 7 μM and iii) compounds which were ARBs at 7 μM but which were directly antibacterial at 20 μM.

Compounds at 7 μM or 20 μM were also tested in combination with antibiotics at concentrations of 0.125 x MIC. There were few hits which overlapped between species (Figure 1). Most of the compounds which did overlap were known antimicrobials or antiseptics (Tables S5-S10). A number of compounds showed interesting potentiation, and these are discussed further below and in the supplementary file.

Three anthracycline-related molecules, daunorubicin, mitoxantrone and epirubicin showed potentiation with one or more combination of drug and species (Table 1). The pattern of activity differed between the three molecules tested, with no evidence of direct antibacterial activity, but differing levels of potentiation for other antibiotics.

Several nucleotide/nucleoside analogues, identified as antimetabolites and/or antiviral agents, also showed potentiation with one or more antibiotic (Table 1). Whilst simplistically such molecules might be expected to have similar effects, via interference with DNA/RNA metabolism in the cell, there were clear differences in the spectrum of activity between the compounds.

Two psychoactive compounds, fluspirilene and oxethazaine were also found to act as ARBs with colistin and merited further investigation, given the possibility that their mode of action might be

different to cationic compounds identified previously as able to potentiate colistin (for example pentamidine [10], which was not found to potentiate colistin activity in this study, and cysteamine, which was not included in this study [11]). The MIC of colistin alone, and in combination with set concentrations of fluspirilene and oxethazaine was determined as above, but in non-cation adjusted Mueller-Hinton broth (Oxoid) and polypropylene plates, incubated for 20 hours at 37°C [12].

Colistin potentiation by fluspirilene and oxethazaine in a wider panel of colistin-resistant strains of *K. pneumoniae* and a smaller number of other Gram-negative pathogens was tested as examples of compounds which were clear ARBs with very little direct antimicrobial activity (Table S3). The studies were designed as a fixed concentration synergy experiment, looking for ARB activity. Initially, MICs and growth curves were used to analyse direct effects of the two compounds. In most cases the MIC was >160 µM for *Klebsiella* spp. and *P. aeruginosa* isolates. For *E. coli*, all strains had an MIC of 160 µM or above for oxethazaine, but two strains (LEC001 and 319238/UR) had MICs of 80 µM for fluspirilene. The notable exception to the high MIC values identified, were the *A. baumannii* strains, which showed an MIC of 20 µM for both oxethazaine and fluspirilene in both colistin-resistant strains (Table S4).

Despite being ARB hits with the original colistin-resistant *K. pneumoniae* strain used in the HTCS, within the broader panel of *Klebsiella* isolates, there were few examples of clear colistin potentiation with either compound. Only strains NCTC 13439 CST 2A (4-fold), MGH 78578 CST A (8-fold) and m109 CST 1B (32-fold) showed greater than 2-fold potentiation of colistin with fluspirilene (Figure 2, Table S3) and no strains showed this level of potentiation with oxethazaine.

In contrast, fluspirilene showed potentiation of colistin in all of the other Gram-negative species tested, with levels ranging from 4-fold (*A. baumannii* W1 CST_R) to >128 fold (*E. coli* LEC001). The latter strain was also the only strain which showed potentiation with oxethazaine, again with increased susceptibility to colistin of >128 fold. Whether derivatives of fluspirilene merit further investigation as a stand-alone antibiotic or as an ARB, may depend on the novelty of its mechanism

of action. The developability is hampered by the relatively high concentration required to achieve potentiation of colistin, for example, around 20 µM against *K. pneumoniae* (equivalent to 9.5 mg/L) compared to the daily dose (10 mg i.m. per day).

The current screen, in line with many other studies, suggests that there might be very few licensed drug compounds which could be simply repositioned, and which would have immediate benefit as adjunct therapies. This does not preclude future studies, looking at other antimicrobial strategies, such as, biofilm disruption [5], anti-virulence compounds [13] or efflux pump inhibition [14], but it does suggest that such studies must be carefully designed to generate useful information. The screening of existing approved drugs, while attractive from a regulatory standpoint and rapid route to market, does not directly address challenges of antimicrobial drug development, including the permeability issue which impacts on drug uptake into Gram-negative bacteria [15], nor the relatively limited chemical space inhabited by most classical drugs [16].

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Table 1: Structures and antimicrobial profiles of interesting hits from the screen. Shaded boxes illustrate direct or ARB activities, in μM, of compounds in combination with meropenem (MEM), ciprofloxacin (CIP), gentamicin (GEN), tigecycline (TGC) or colistin (CST) in the four Gram-negative species tested. Where compounds had activity at both 20 μM and 7 μM, only 7 μM is represented in the table.

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Anthracyclines						Antimetabolites						Psychoactive						Miscellaneous					
		A. baumannii	*K. pneumoniae*	*E. coli*	*P. aeruginosa*			*A. baumannii*	*K. pneumoniae*	*E. coli*	*P. aeruginosa*			*A. baumannii*	*K. pneumoniae*	*E. coli*	*P. aeruginosa*			*A. baumannii*	*K. pneumoniae*	*E. coli*	*P. aeruginosa*
Daunorubicin	Direct					Zidovudine	Direct		20	7		Gabapentin	Direct					Thonzonium bromide	Direct				
	MEM						MEM		7				MEM			20			MEM				
	CIP						CIP						CIP			20			CIP				
	GEN						GEN		7				GEN						GEN				
	TGC						TGC						TGC						TGC				
	CST	20	20				CST	7	7				CST						CST	7	7		
Doxorubicin	Direct					Didanosine	Direct		20			Thioridazine	Direct					Pyrvinium pamoate	Direct				
	MEM						MEM		7				MEM						MEM			7	
	CIP						CIP						CIP						CIP				
	GEN						GEN						GEN						GEN			7	20
	TGC						TGC						TGC						TGC				
	CST						CST	20	20				CST	20					CST	7			
Mitoxantrone	Direct					Floxuridine	Direct		20	7		Oxethazaine	Direct					Auranofin	Direct	7	20		7
	MEM						MEM		7				MEM	20					MEM		7	20	
	CIP						CIP		7				CIP	20					CIP		20	20	
	GEN						GEN						GEN	20					GEN		7		
	TGC				20		TGC						TGC	20					TGC				
	CST						CST	20	7				CST	7	20				CST		7		
Epirubicin	Direct					Gemcitabine	Direct					Fluspirilene	Direct	20									
	MEM						MEM						MEM										
	CIP						CIP			20			CIP										
	GEN						GEN						GEN										
	TGC						TGC						TGC										
	CST	20	20				CST						CST	7	20								

221 **Figure 1: Few ARB hits show any conservation cross-species or with specific antibiotics.** Heat map showing ARB hits by species and antibiotic potentiated,
222 coloured according to the amount of growth inhibition they caused in each species in combination with each antibiotic. (grey is where the combination was
223 not tested).

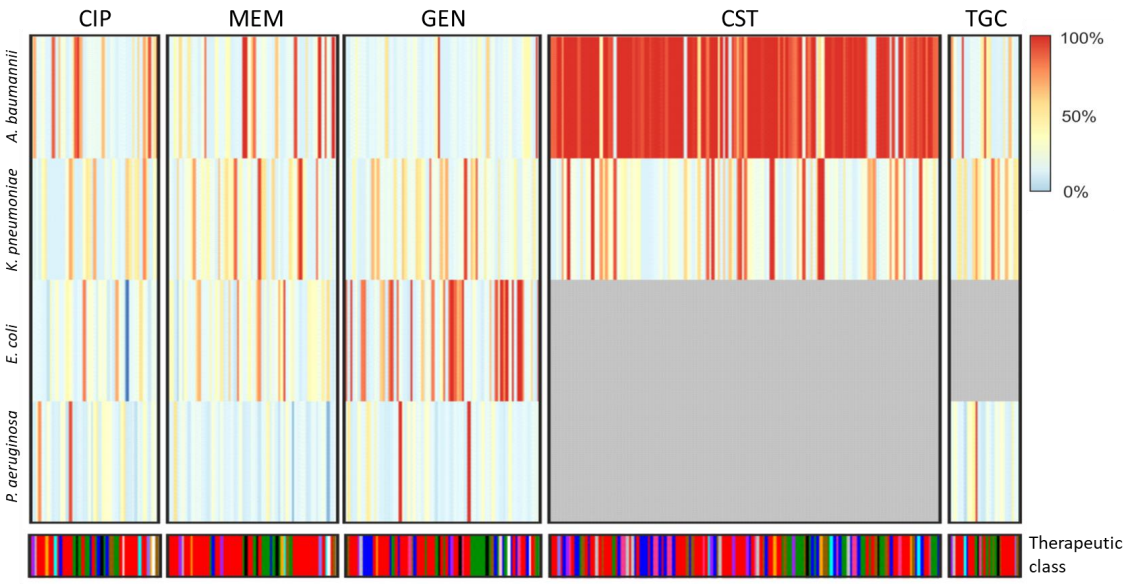

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Figure 2: Colistin ARB potential of fluspirilene. A wider panel of colistin-resistant strains were tested in the presence of fluspirilene. Although the *K. pneumoniae* strain used in the HTCS showed colistin-potentiation by fluspirilene, this was not reflected in a wider panel. However, fluspirilene did potentiate colistin in other Gram-negative species. Arrows on the *K. pneumoniae* panel indicate the change in MIC for two specific strains. This highlights an example where fluspirilene is antagonistic to colistin but where the MIC is in the same range as some strains where potentiation is observed.